APPLICABILITY OF DNA BARCODING TO MUSEUM SPECIMENS OF BIRDS FROM THE DEMOCRATIC REPUBLIC OF THE CONGO

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Abstract. The ornithological collections of the Royal Museum for Central Africa in Tervuren and the Royal Belgian Institute of Natural Sciences in Brussels contain approximately 155 000 specimens collected in the Democratic Republic of the Congo (DRC). They include type specimens and other samples from historical populations that represent an exceptional source of information for exploring how habitat fragmentation due to deforestation or global climate changes affect patterns of biodiversity in African birds. By attempting to obtain DNA sequences from these archive collections we intend to make them useful for genetic studies and to contribute to a reference library of DNA sequences, thus allowing the future identification of Central African bird species through DNA barcodes. Our project aims to sequence approximately 950 museum specimens, representing 225 species, collected between 1845 and 2008. Our preliminary results reveal that the degradation of DNA in most museum specimens does not allow the amplification of the standard DNA barcode fragment (694 bp). Nevertheless, we have been able to sequence shorter fragments (298 bp and 100 bp) for the majority of the selected specimens, implying that the collections in the RMCA and the RBINS contain DNA information that remains useful for barcoding purposes. More elaborate experiments might yield longer DNA sequences for phylogenetic and phylogeographic studies.

Key words: Democratic Republic of the Congo, DNA barcoding, COI, Aves, museum sample, archival DNA.

INTRODUCTION

Natural history collections hold unique and irreplaceable samples of historical populations. Museum specimens play a crucial role in the study of biodiversity, biological invasions, habitat loss, global climate change, pathogens and disease vectors as well as environmental contaminants (Suarez & Tsutsui 2004). Early publications demonstrated that genetic information from museum specimens adds an accurate historic reference to the study of extant populations (Thomas *et al.* 1990, Roy *et al.* 1994). More recently, comparisons between extant biomaterial and archival DNA have contributed to the identification of living descendants of presumably extinct species e.g. a Galapagos tortoise (Poulakakis *et al.* 2008) and the giant sable antelope (Pitra *et al.* 2008). The genetic analysis of historical mammal specimens has also clarified the phylogenetic position of the extinct Alpine lynx (Gugolz *et al.* 2008), the quagga (Leonard *et al.* 2005), three tenrec genera (Asher & Hofreiter 2006), and the taxonomic status of the extinct kouprey (Bovinae) (Hassanin & Ropiquet 2004, 2007). At a population level, archival DNA has enabled the direct measurement of the genetic variability in a pre-bottleneck population of the greater prairie chicken (Bouzat *et al.* 1998). The information on historical genetic variability may allow the estimation of past population sizes (Ramakrishnan *et al.* 2005), useful for wildlife conservation (Leonard 2008). More resolved phylogenies have been obtained using complete mitochondrial genomes of the Tasmanian tiger (Miller *et al.* 2009) and Mediterranean tortoises (Parham *et al.* 2006), or using nuclear DNA from old bird collections (Irestedt *et al.* 2006).

In addition to their historical value, museum samples represent an essential link between biological data and taxonomic knowledge and provide a means to verify the taxonomic identity of the sequenced specimens (Pleijel *et al.* 2008). For example, using DNA sequences from type specimens, Stuart *et al.*

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TABLE 1. Detail of the number of samples, number sequenced, and sequences used. Percentages are given between parentheses in comparison with the sampling effort.

	Sampling effort	Sequenced				Not sequenced	Sequences used[b]
		100 bp	298 bp	694 bp	Total	0 bp	
Specimens (total)	951	326 (34%)	313 (33%)	82 (9%)	721 (76%)	230 (24%)	621 (65%)
RMCA	667	187 (28%)	236 (35%)	52 (8%)	475 (71%)	192 (29%)	386 (58%)
RBINS	256	139 (54%)	76 (30%)	5 (2%)	220 (86%)	36 (14%)	215 (84%)
UCZM	19	0	0	17 (90%)	17 (90%)	2 (10%)	14 (74%)
WML	1	0	1 (100%)	0	1 (100%)	0	1 (100%)
MBC[c], Korea	8	0	0	8 (100%)	8 (100%)	0	5 (62%)
Species[3]	225	132 (59%)	121 (54%)	18 (8%)	186 (83%)	39 (17%)	177 (79%)
Genera	84	57 (68%)	48 (57%)	4 (5%)	75 (89%)	9 (11%)	69 (82%)
Families	45	31 (69%)	34 (76%)	2 (4%)	41 (91%)	4 (9%)	38 (84%)
Orders	13	12 (92%)	8 (62%)	2 (15%)	13 (100%)	0	13 (100%)

[1] Only sequences that have a minimum 100 bp overlap with the standard (Folmer *et al.* 1994) and the universal mini-barcode (Meusnier *et al.* 2008) are used.

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[3] Only specimens identified at the species level are taken into account.

(2008) were able to recognize misidentifications in incongruent studies on the diversity of Asian leaf turtles. Voucher specimens therefore constitute necessary reference material for the Barcode of Life Initiative, which aims to sequence a standard DNA fragment of the cytochrome *c* oxidase subunit I gene (COI) for all animal species (Hebert *et al.* 2003). This approach requires a DNA reference library as comprehensive and accurate as possible in order to be useful for species identification (Meyer & Paulay 2005). Previous studies, in the context of the All Birds Barcoding Initiative (ABBI), have demonstrated the effectiveness of COI barcodes to identify North American (Kerr *et al.* 2007), Korean (Sook *et al.* 2006), and Neotropical bird species (Kerr *et al.* 2009). Although most avian DNA barcodes were obtained from fresh samples, DNA barcoding of birds from museum collections has already been shown to be useful in determining the taxonomic relationship between living and extinct or endangered species, e.g. quails in New Zealand (Seabrook-Davison *et al.* 2009) or the ivory-billed woodpecker (Fleischer *et al.* 2006).

However, obtaining DNA sequences from museum specimens is often a challenge. DNA molecules become increasingly fragmented with time. Fixation treatments and storage conditions severely affect the DNA extraction process and downstream applications. Finally, tissue sampling methods should be adapted in order to safeguard the integrity of the museum specimens (Wandeler *et al.* 2007). In order to address some of these difficulties, a number of protocols has been developed for DNA extraction of archival samples in general (Rohland *et al.* 2004, Rohland & Hofreiter 2007), from feathers (Horvath *et al.* 2005), or from toe-pads of dried bird skin collections (Mundy *et al.* 1997). Furthermore, depending on the collections, the specimens, and even the type of tissues, DNA appears to be very variable in quality and quantity (Wandeler *et al.* 2007).

The bird collections of the Royal Museum for Central Africa in Tervuren (RMCA) and the Royal Belgian Institute of Natural Sciences in Brussels (RBINS) hold *ca.* 155 000 well-documented voucher specimens from the Democratic Republic of the Congo (DRC). Some of these originate from areas that are difficult to access or from historical populations that might have become strongly reduced or even extinct. They also include type specimens, which represent the ultimate reference material for species identification and which testify to the existence of taxa in an area for which the fauna is poorly known (Louette *et al.* 2010). The existence of these two important collections is an important opportunity, especially when compared to regions such as the Indomalayan region, where the past and current ornithological

sampling effort has been poor and where only a small proportion of its bird fauna is represented in vouchered tissue collections (Sodhi *et al.* 2007). In view of the arguments above, this study looks at the potential value of the bird collections of the RBINS and the RMCA for DNA barcoding.

MATERIAL AND METHODS

The number of specimens sampled was 951 (RMCA: 667, RBINS: 256, UCZM: 19, WML: 1, Korea National Park Service: 8). They represent 225 morphologically identified species belonging to 84 genera (Table 1). This exploratory study focused on genera where a morphological taxonomic expertise was available within the institutions concerned (e.g. *Accipiter* and *Ploceus*). On average, we selected four specimens per species (Appendix). Thirty-seven species are represented by a single specimen, but some species of the genera *Accipiter* and *Ploceus* were intensively sampled (up to 46 specimens per species). Reliably identified specimens (adult specimens with typical morphology) were selected from different geographic localities. In addition, some unreliably identified specimens (e.g. juveniles or individuals with a peculiar morphology) were used to test the effectiveness of DNA barcoding in detecting possible misidentifications.

Tissue sampling, and pre- and post-PCR manipulations were performed in different rooms in order to prevent contamination. Further, we never processed conspecific samples simultaneously in order to allow the detection of cross-contamination between samples in adjacent tubes. Tissue samples were taken from toe-pad skins (Mundy *et al.* 1997), lysed overnight, and total genomic DNA was extracted using NucleoSpin tissue kits (Macherey-Nagel). Moreover, total DNA yield was visualized by gel electrophoresis for a set of samples of different ages in order to estimate the size-range of DNA fragments in the DNA extracts. In order to be able to amplify DNA fragments from highly degraded samples, we attempted to amplify different fragment sizes using three primer combinations that always involved the same forward primer (Fig. 1). To achieve

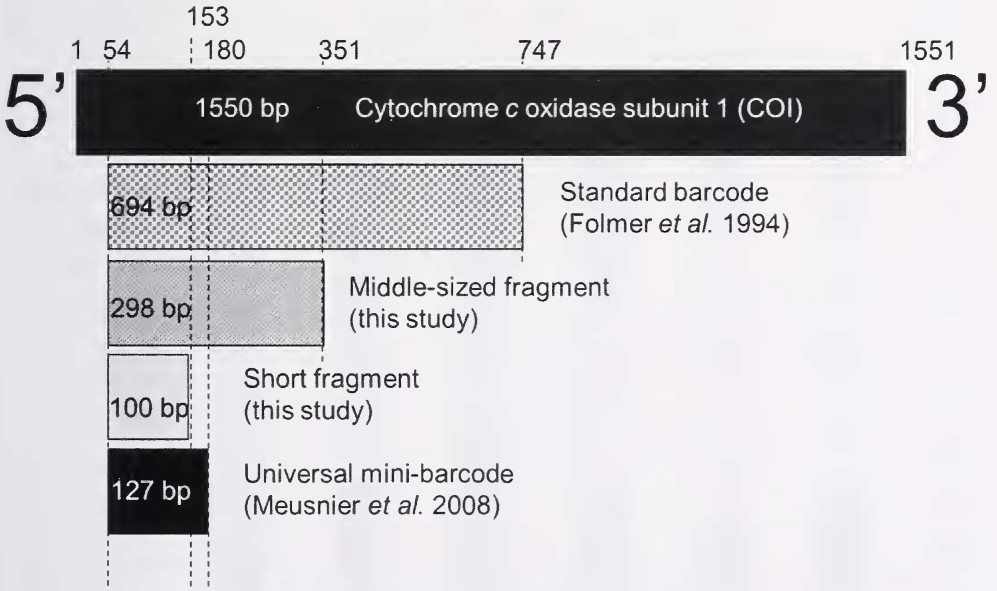

FIG. 1. Relative position of the different DNA fragments amplified and sequenced in this study on the cytochrome *c* oxidase subunit 1 (COI) gene sequence of *Buteo buteo* (GenBank accession number NC_003128) and the universal mini-barcode (in black). Relative nucleotide positions on the COI gene fragment are given at the top of the figure. Lengths (bp) of the fragments are given in the respective rectangles representing the fragments.

this goal we used the degenerated primers BirdF1d (5'-TCAACCAACCACAAAGAYATYGGYAC-3') and BirdR1d, (5'-ACGTGGGAGATGATTCC-GAAKCCKGG-3') modified after Hebert *et al.* (2004) and Lohman *et al.* (2008) for the standard DNA barcode region (694 bp); BirdF1d and Bird-H351d (5'-CCTGCTCCWGCTTCTAYDGT-3') to amplify a 298 bp DNA fragment corresponding to the first half of the standard DNA barcode, and BirdF1d together with BirdH153d (5'-ACGATTA-CRTTGTARATYTGRTC-3') to amplify a short DNA fragment (100 bp) representing the first part of the standard DNA barcode. This "mini-barcode" region corresponds to the first 100 bp of the universal DNA mini-barcode (127 bp) proposed by Meusnier *et al.* (2008).

After preliminary tests failed to amplify the standard DNA barcode for most samples, we targeted the middle-sized fragment (298 bp) to obtain the best balance between sequence length and number of successful amplifications. Finally, we also tried to amplify the 100 bp fragment for the samples that failed in the previous PCR assays. We evaluated the DNA quantity and quality of the 248 samples from RBINS with a ND-1000 Spectrophotometer (Nano-Drop) to be able to use a constant final concentration of *ca.* 3 ng/µl DNA template in each PCR. The amplified fragments were directly sequenced using an ABI 3130xl Genetic Analyzer (Applied Biosystems). DNA sequences were checked for quality and aligned by hand. Since Sefc *et al.* (2007) demonstrated the occurrence of single base errors (double peaks and artifact substitutions) in PCR products obtained from museum bird specimens, we read all double peaks as ambiguity codes. We calculated K2P distances using MEGA 4 (Tamura *et al.* 2007) and used TaxonDNA v.1.5a12 (Meier *et al.* 2006) to cluster sequences based on pairwise distances and check the accordance between these clusters and the identification of the voucher specimens.

RESULTS

DNA sequencing. The systematic DNA barcoding of 951 museum specimens representing 225 morphologically identified species belonging to 84 genera produced 721 sequences (76% of the sampled specimens). Forty-three percent of them correspond to the middle-sized fragment, 45% to the short fragment, and 11% to the standard DNA barcode fragment (Table 1). The effect of the age of the specimen collection on the results (Fig. 2.) shows that even if

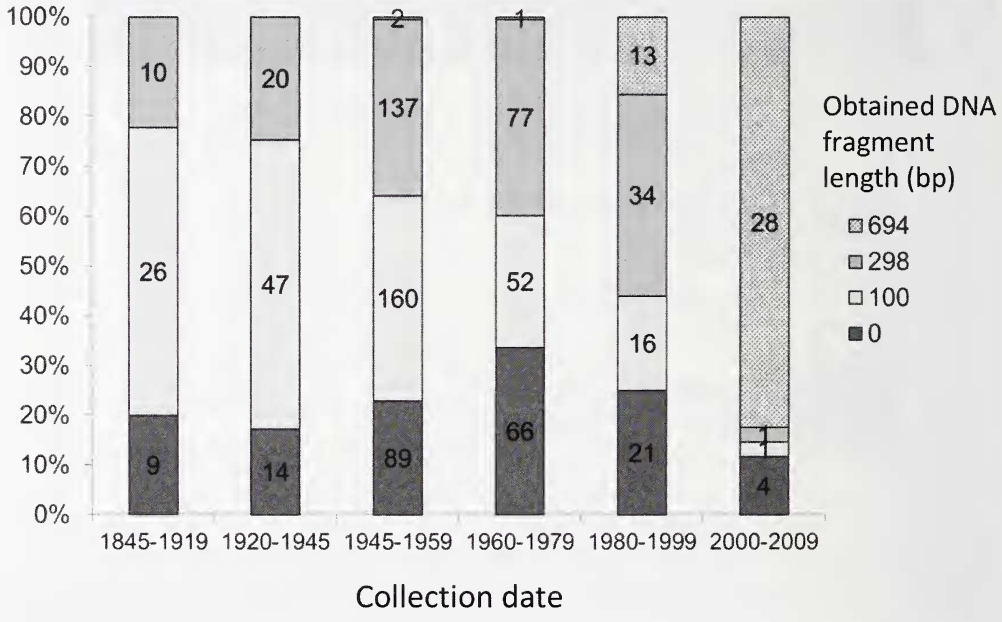

FIG. 2. Histogram showing the effect of the collection date on the success rate of obtaining different sizes of DNA sequences. Absolute numbers are given in the columns.

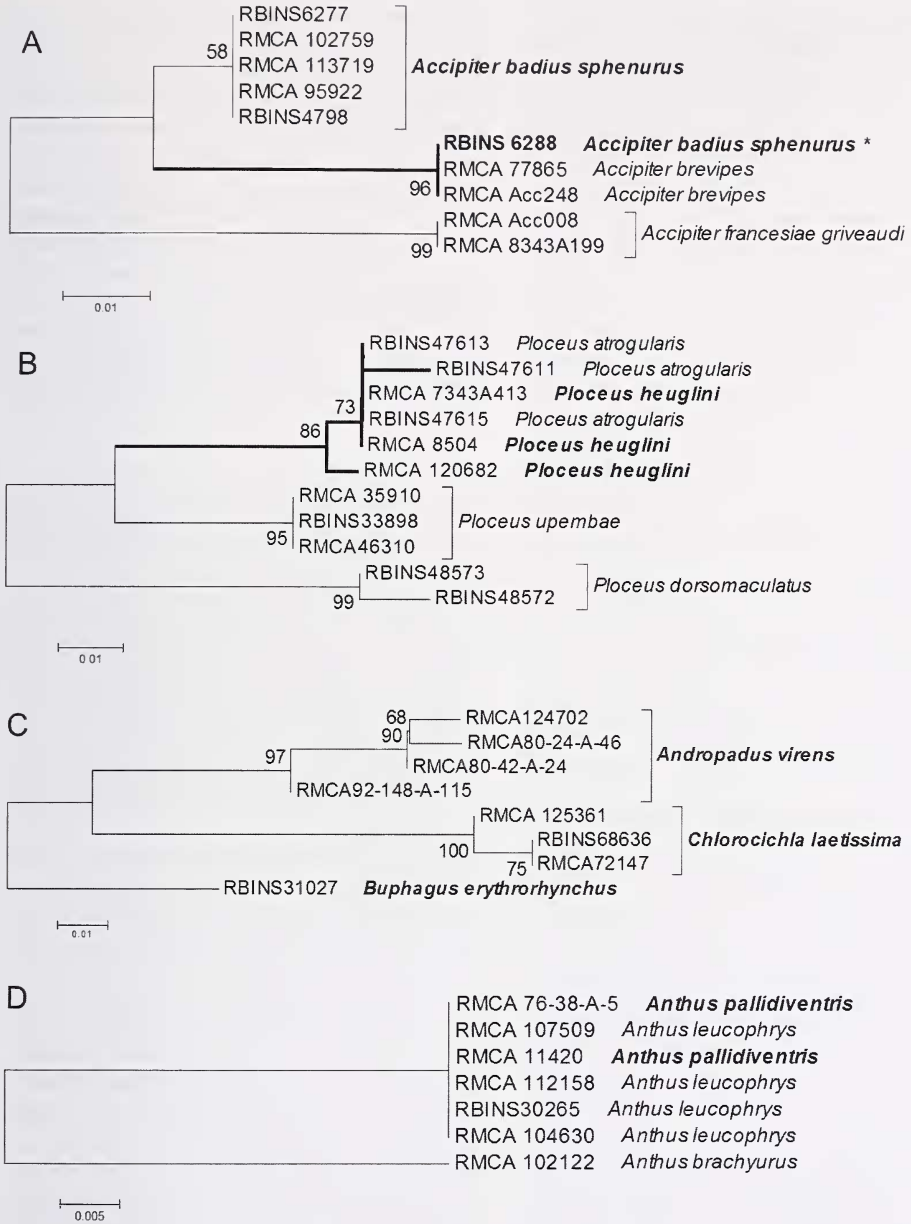

FIG. 3. Neighbor-joining trees constructed with K2P genetic distances of a selection of DNA (mini-) barcodes. A. Mislabeling: specimen No. RBINS 6288 was labeled years ago as *Accipiter badius sphenurus* but its COI fragment is similar to those of specimens identified as *Accipiter brevipes*; identification was corrected after close inspection showed the skin was indeed referable to *Accipiter brevipes*. B. Synonymy: specimens labeled as *Ploceus atrogularis* and *P. heuglini* have COI sequences that cluster together in one clade. It appears that different synonymic species names were applied in the different collections. C. Intraspecific variation in 100 bp sequences from specimens identified as *Andropadus virens* and *Chlorocichla laetissima*. D. Sequences from specimens identified as *Anthus pallidiventris* and *Anthus leucophrys* cluster together in one clade.

the overall success rate of obtaining a sequence varies from 66% to 88%, the proportion of short fragments (100 bp) versus middle-sized fragments (298 bp) increases with the age of the voucher specimens. It is important to point out that we detected no frameshifts or stop codons, which might have indicated the presence of nuclear mitochondrial DNA (numt). Double peaks were found at a rate of 10^{-4} per base pair, i.e. three times less than the $3x10^{-4}$ per bp found by Sefc *et al.* (2007).

We assembled a data set of 621 sequences that had a minimum overlay of 100 bp with the standard (Folmer *et al.* 1994) and the universal mini-barcode (Meusnier *et al.* 2008). Based on initial morphological identifications, they represent 177 species (i.e. 79% of the selection) belonging to 69 genera (i.e. 82% of the selection) (Table 1). On average, we obtained 2.9 sequences per species (range: 0-34). Sixty-four species belonging to the genera *Accipiter* and *Ploceus* were sampled more intensively. In contrast, 62 species (28% of the selection) are represented by a single sequence and cannot therefore be validated by conspecific sequences.

DNA barcoding. Two neighbor-joining trees based on pairwise K2P distances between all 621 sequences were done with complete and pairwise deletion. Both trees distinguish clusters of similar sequences that allowed the correct taxonomic assignment of the unreliably identified specimens. In a few cases, misidentifications (e.g. by mislabeling) were revealed (Fig. 3A.). In contrast, some cases of unexpectedly low genetic divergences between specimens with different species names agree with their taxonomic status as synonyms (Fig. 3B.). On the genus level, our sequences allow the correct allocation of 65 of the 69 genera for which a sequence is available. Nevertheless, we have to consider that the resolution of the resulting tree depends on the taxonomic groups and the fragment length considered. Based on the 100 bp sequences, genera *Phodilus* and Pseudochelidon (represented in our sampling by species *Phodilus prigoginei* and *Pseudochelidon eurystomina*) cannot be distinguished like in the case of *Malimbus* and *Ploceus*. On the species level, sequences of 122 morphologically identified species cluster unambiguously in the neighbor-joining tree. Even intraspecific variability is visible in the 100 bp sequences of some species (e.g., Fig. 3C.). The other 55 species are lumped together with sequences of other species (e.g. Fig. 3D.). Therefore, species identification based on the obtained DNA barcodes of our bird collections appears to be only accurate in 69% of the cases. However, most species with an ambiguous DNA barcode belong to the genera *Ploceus* (Ploceidae), *Anthus* (Motacillidae), and *Indicator* (Indicatoridae), taxonomically the most complex groups in our data set. The proportion of species identified morphologically that can unambiguously be distinguished by the DNA barcodes is very variable among genera, ranging from 50% for the genus *Ploceus* to 100% (e.g. for the genus *Ardeola*). Here we emphasize that identifications of voucher specimens are reliable and this imprecision is thus most probably due to the restricted set of characters present in the sequences obtained.

DISCUSSION

This DNA barcoding approach showed that short DNA sequences can be obtained at a reasonable rate of success. For our collections, the amplification of the middle-sized fragment appears to be an adequate approach for specimens collected in the past 50 years. It is short enough to get a satisfactory success rate (> 45%) and still allows more information to be obtained than from the short fragment. For specimens older than 50 years we recommend targeting the short fragment for a better success rate (> 57%). These results confirm that the minimalist barcode approach increases the overall success rate when sequencing degraded DNA from museum specimens (Hajibabaei *et al.* 2006, Meusnier *et al.* 2008).

The success rates found here are based on two different collections and may depend on the tested species since, for example, the size of the tissue sample used for DNA extraction correlates with the size of the bird specimen. Therefore these rates may serve as an indication for other projects or collections but cannot replace an exploratory experiment on the collection under study. Because preservation methods and conservation conditions may vary considerably in the usage of fixative agent, the temperature and the humidity in the collection room or the different handlings made by researchers, etc., DNA quality can be significantly different even among samples of similar age (Wandeler *et al.* 2007).

In our study, standard protocols (i.e. DNA extraction kits, standardized PCR conditions) were used. The only alteration was the use of three different combinations of degenerated primers. This implies that further improvements of the procedure, such as a more efficient DNA extraction method (e.g.

phenol-chloroform) or manipulations at the PCR stage (e.g. amplifications with primers more specific to the group of interest, testing different polymerases, reamplifications of PCR products), will probably increase the success rate. Unfortunately, testing all possible improvements during this pilot study would have been too expensive and too time-consuming. Alternatively, the overlapping of short fragments allows the assembly of larger fragments. In addition, next-generation sequencing technologies that are able to read billions of short fragments per run (Metzker 2010) also offer excellent opportunities to work with degraded DNA. The results presented here are, from this point of view, promising for the utilization of bird specimens from museum collections as a source of historical genetic information.

The DNA barcodes produced are of great importance for the All Bird Barcoding Initiative since they concern birds from the Afrotropical region, a region where only 587 of an estimated total of 2384 species are considered to be barcoded (ABBI 2011). Nonetheless, we have to consider that the barcodes produced only give a partial indication of the intraspecific genetic variation of the species because the sampling covers only part of their entire geographic range. Ideally, the sampling has to take the evolutionary history of the species into account (Zhang *et al.* 2010). This precaution is all the more relevant since several studies have shown a high level of intraspecific diversity in African birds that are widely distributed in montane regions (Bowie *et al.* 2006, Kahindo *et al.* 2007) as well as in lowland rainforests (Marks 2010). For example, in our set of sequences we observed a single haplotype with the COI marker (298 bp) in three specimens of *Platysteira peltata* collected in two different localities in the Upemba National Park (DRC, Katanga) whereas Njabo *et al.* (2008) discovered a substantial haplotype diversity with marker ND2 (1041 bp) in this species with a broad geographic distribution. Substantial genetic divergences were also observed over a short distance as revealed by Voelker *et al.* (2010) with *Cossypha anomala* in tropical montane areas where a diversification of the Africa's forest avifauna seems to take place (Fjeldså & Bowie 2008). At this stage, it is premature to draw definitive conclusions on the species identification efficiency of the resulting DNA barcodes because the relatively large proportion of ambiguous DNA barcodes observed can be due to both DNA (mini-)barcoding and taxonomical artifacts. First, the limited size of the mini-barcodes may lack the information necessary to distinguish a large number of well-differentiated species (Hajibabaei *et al.* 2006, Wiemers & Fiedler 2007). This probably explains the lower percentage of species correctly delineated by the COI marker compared to studies using the standard barcode fragment (Aliabadian *et al.* 2009). Secondly, in some animal groups with numerous species and where morphology is less variable, taxonomic knowledge can overlook the presence of multiple taxa within a traditional species (overlumping). Alternatively, different morphotypes can inappropriately be recognized as individual species (oversplitting) (Funk & Omland 2003). However, in birds several studies have shown that even recently diverged sister species pairs have fixed nucleotide substitutions that can serve as diagnostic characters (Tavares & Baker 2008, Kerr *et al.* 2009). These hypotheses will have to be tested in every taxon where we observe discrepancies between DNA barcoding and morphology. There is no doubt that this first phase of DNA barcoding, even when done in a systematic manner, raises scientific questions that require a second phase of more detailed investigations in close collaboration with bird taxonomists.

In summary, the application of DNA barcoding to avian collections from the DRC provided (i) information on the DNA quality of the museum collection, as it is feasible to obtain fragments of 100-300 bp, (ii) new DNA barcodes for birds from the Afrotropics, where the estimated proportion of barcoded bird species is currently the lowest of all biogeographical regions (ABBI 2011), and (iii) species identification based on the DNA barcodes which contributed to the correction of the species assignment of some specimens in the collections and raised taxonomic questions that need to be addressed in close collaboration with taxonomists.

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APPENDIX. List of all species under study. (Ns) Number of sampled specimens. (Nsq) Number of sequences with minimum 99 bp overlap with the universal mini-barcode. Highlighted species names may differ from one reference to another.

Species	Ns.	Nsq.	Accession numbers
Accipiter badius	24	12	JF312106 JF312127 JF312113 JF312120 JF312110 JF312111 JF312088 JF312119 JF312136 JF312105 JF312090 JF312159
Accipiter brevipes	4	4	JF312114 JF312152 JF312109 JF312192
Accipiter castanilius	4	1	JF312150
Accipiter erythropus	3	1	JF312092
Accipiter francesiae	46	34	JF312089 JF312196 JF312098 JF312179 JF312176 JF312175 JF312169 JF312099 JF312097 JF312104 JF312086 JF312163 JF312167 JF312168 JF312093 JF312094 JF312171 JF312162 JF312177 JF312164 JF312165 JF312166 JF312178 JF312174 JF312173 JF312172 JF312170 JF312096 JF312189 JF312100 JF312101 JF312102 JF312103 JF747507
Accipiter gentilis	2	2	JF312084 JF312195
Accipiter gularis	2	2	JF312215 JF312115
Accipiter madagascariensis	2	1	JF312117
Accipiter melanoleucus	4	3	JF312091 JF312187 JF312188
Accipiter minullus	11	10	JF312137 JF312147 JF312133 JF312123 JF312116 JF312158 JF312211 JF312212 JF312213 JF312207
Accipiter nisus	12	6	JF312087 JF312085 JF312161 JF312190 JF312191 JF312194
Accipiter ovampensis	2	1	JF312121
Accipiter rufiventris	3	1	JF312144
Accipiter soloensis	7	4	JF312185 JF312184 JF312181 JF312182
Accipiter tachiro	29	15	JF312143 JF312202 JF312214 JF312197 JF312210 JF312209 JF312198 JF312208 JF312205 JF312201 JF312204 JF312199 JF312200 JF312203 JF312142
Accipiter toussenelii	14	2	JF312129 JF312151
Accipiter virgatus	1	1	JF312186
Afropavo congensis	1	0	n.a.
Agapornis swindernianus	1	1	HQ997940
Alethe poliocephala	3	2	HQ997979 HQ998071
Anaplectes rubriceps	3	1	HQ998336
Andropadus virens	4	4	HQ998164 HQ998272 HQ998275 HQ998298
Anomalospiza imberbis	6	6	HQ998036 HQ998057 HQ998344 HQ998404 HQ998415 HQ998432
Anthus bocagei	3	0	n.a.
Anthus brachyurus	5	1	HQ998117
Anthus cinnamomeus	9	5	HQ998185 HQ998196 HQ998197 HQ998246 HQ998252
Anthus itombwensis	3	1	HQ998304
Anthus katangae	2	2	HQ998176 HQ998224
Anthus lacuum	3	2	HQ998203 HQ998206
Anthus latistriatus	3	2	HQ998287 HQ998303
Anthus leucophrys	17	4	HQ998004 HQ998122 HQ998135 HQ998143
Anthus lineiventris	4	1	HQ998008

APPENDIX. Continued.

Species	Ns.	Nsq.	Accession numbers
Anthus pallidiventris	5	2	HQ998148 HQ998259
Anthus similis	14	2	HQ998145 HQ998175
Anthus vaalensis	9	5	HQ998005 HQ998006 HQ998007 HQ998190 HQ998225
Apalis alticola	3	3	HQ997986 HQ997987 HQ997988
Apalis goslingi	2	2	HQ998120 HQ998124
Apalis kaboboensis	2	2	HQ998253 HQ998293
Apalis porphyriolaema	3	3	HQ998180 HQ998288 HQ998316
Apalis schoutedeni	1	0	n.a.
Apus caffer	1	1	HQ997974
Ardeola idae	6	5	HQ998184 HQ998202 HQ998219 HQ998226 HQ998227
Ardeola ralloides	12	9	HQ998156 HQ998186 HQ998192 HQ998194 HQ998204 HQ998215 HQ998234 HQ998277 HQ998294
Ardeola rufiventris	3	1	HQ998317
Batis erlangeri	4	4	HQ998150 HQ998267 HQ998302 HQ998305
Batis minor	1	1	HQ998315
Batis molitor	3	3	HQ997992 HQ997993 HQ997994
Brachycope anomala	3	2	HQ998357 HQ998424
Bradypterus cinnamomeus	3	2	HQ997984 HQ997985
Bubulcus ibis	5	1	HQ998116
Buphagus africanus	1	1	HQ998050
Buphagus erythrorhynchus	2	1	HQ998012
Butastur rufipennis	2	1	HQ998257
Buteo buteo	1	0	n.a.
Butorides striatus	2	1	HQ998236
Caprimulgus prigoginei	1	1	HQ998268
Centropus monachus	7	7	HQ997943 HQ997944 HQ997945 HQ997946 HQ997947 HQ997948 HQ997949
Chlorocichla laetissima	4	4	HQ998101 HQ998167 HQ998230 HQ998242
Chlorocichla prigoginei	3	3	HQ998159 HQ998254 HQ998292
Circus aeruginosus	1	0	n.a.
Circus cyaneus	1	0	n.a.
Coracina azurea	2	0	n.a.
Coracina caesia	3	1	HQ998239
Coracina graueri	5	2	HQ997972 HQ997973
Coracina pectoralis	2	2	HQ998130 HQ998220
Cossypha bocagei	3	3	HQ997976 HQ997977 HQ997978
Cuculus canorus	2	2	HQ997941 HQ997942
Dendropicos goertae	5	4	HQ998201 HQ998269 HQ998279 HQ998282
Dendropicos griseocephalus	3	3	HQ998245 HQ998263 HQ998281
Dendropicos sp.	2	2	HQ998136 HQ998189
Estrilda astrild	10	0	n.a.
Estrilda atricapilla	4	4	HQ998333 HQ998335 HQ998407 HQ998411
Estrilda nigriloris	1	1	HQ998013

APPENDIX. Continued.

Species	Ns.	Nsq.	Accession numbers
Euplectes hartlaubi	3	2	HQ998332 HQ998361
Foudia eminentissima	1	0	n.a.
Francolinus afer	14	9	HQ997925 HQ997928 HQ998372 HQ998376 HQ998391 HQ998403 HQ998405 HQ998414 HQ998419
Francolinus albogularis	5	3	HQ997930 HQ997931 HQ997932
Francolinus camerunensis	3	3	HQ998367 HQ998378 HQ998418
Francolinus coqui	6	5	HQ997929 HQ998350 HQ998366 HQ998369 HQ998394
Francolinus kasaicus	2	0	n.a.
Francolinus lathami	7	3	HQ998088 HQ998089 HQ998417
Francolinus levaillantii	6	3	HQ997933 HQ997934 HQ998321
Francolinus nahani	3	2	HQ998326 HQ998380
Francolinus nobilis	1	1	HQ998110
Francolinus ruandae	2	2	HQ998389 HQ998390
Francolinus shelleyi	1	1	HQ997926
Francolinus sp.	1	1	HQ998371
Francolinus squamatus	17	15	HQ997935 HQ997936 HQ997937 HQ998330 HQ998341 HQ998353 HQ998362 HQ998368 HQ998382 HQ998383 HQ998397 HQ998412 HQ998416 HQ998420 HQ998425
Glaucidium albertinum	2	2	HQ998216 HQ998223
Glaucidium capense	2	0	n.a.
Graueria vittata	2	2	HQ998163 HQ998248
Guttera plumifera	1	1	HQ998082
Gymnobucco bonapartei	3	0	n.a.
Gymnobucco calvus	2	0	n.a.
Gymnobucco peli	1	0	n.a.
Gymnobucco sladeni	3	1	HQ998138
Hemitesia neumanni	4	2	HQ998160 HQ998238
Indicator conirostris	4	0	n.a.
Indicator exilis	8	3	HQ997961 HQ997962 HQ997963
Indicator indicator	9	5	HQ997953 HQ997954 HQ997955 HQ998153 HQ998232
Indicator maculatus	8	5	HQ998074 HQ998093 HQ998262 HQ998271 HQ998280
Indicator meliphilus	2	2	HQ998237 HQ998313
Indicator minor	9	3	HQ997964 HQ997965 HQ997966
Indicator pumilio	3	0	n.a.
Indicator sp.	4	4	HQ997960 HQ998076 HQ998083 HQ998105
Indicator variegatus	7	5	HQ997950 HQ997951 HQ997952 HQ998152 HQ998243
Indicator willcocksi	4	4	HQ998077 HQ998133 HQ998284 HQ998309
Ixobrychus minutus	2	2	HQ997927 HQ998081
Ixobrychus sturmii	2	1	HQ997924
Kaupifalco monogrammicus	1	0	n.a.
Laniarius barbarus	2	0	n.a.
Linurgus olivaceus	3	0	n.a.
Lioptilus chapini	4	4	HQ998115 HQ998139 HQ998151 HQ998205

APPENDIX. Continued.

Species	Ns.	Nsq.	Accession numbers
Lioptilus rufocinctus	2	1	HQ998212
Lybius minor	3	3	HQ997969 HQ997970 HQ997971
Malimbus cassini	2	2	HQ998324 HQ998428
Malimbus coronatus	2	2	HQ998334 HQ998355
Malimbus nitens	2	2	HQ998346 HQ998406
Malimbus rubricollis	3	3	HQ998123 HQ998137 HQ998142
Melaenornis ardesiaca	3	3	HQ998161 HQ998162 HQ998240
Melichneutes robustus	2	2	HQ998241 HQ998286
Melignomon eisentrauti	1	1	HQ998274
Melignomon zenkeri	5	5	HQ998103 HQ998128 HQ998141 HQ998247 HQ998307
Micronisus gabar	2	1	HQ998233
Micronisus metabates	2	1	HQ998168
Muscicapa aquatica	6	6	HQ997998 HQ997999 HQ998000 HQ998001 HQ998002 HQ998003
Muscicapa lendu	3	3	HQ998118 HQ998289 HQ998290
Muscicapa olivascens	3	3	HQ998126 HQ998214 HQ998318
Nectarinia congensis	3	0	n.a.
Nectarinia humbloti	3	0	n.a.
Nectarinia rockefelleri	3	0	n.a.
Neolestes torquatus	3	3	HQ998140 HQ998266 HQ998306
Parmoptila woodhousi	5	5	HQ998327 HQ998331 HQ998381 HQ998413 HQ998441
Phedina brazzae	3	1	HQ998255
Phodilus prigoginei	1	1	HQ998210
Phyllastrephus lorenzi	3	3	HQ998127 HQ998198 HQ998273
Pitta angolensis	2	2	HQ998072 HQ998073
Platysteira peltata	3	3	HQ997995 HQ997996 HQ997997
Ploceus albinucha	6	3	HQ998199 HQ998209 HQ998228
Ploceus alienus	3	3	HQ998029 HQ998067 HQ998092
Ploceus angolensis	2	2	HQ998356 HQ998363
Ploceus atrogularis	3	3	HQ998054 HQ998055 HQ998056
Ploceus aurantius	15	10	HQ998276 HQ998283 HQ998300 HQ998365 HQ998399 HQ998408 HQ998409 HQ998410 HQ998422 HQ998427
Ploceus baglafecht	6	5	HQ998323 HQ998328 HQ998421 HQ998433 HQ998439
Ploceus bertrandi	1	1	HQ998049
Ploceus bicolor	6	6	HQ998026 HQ998027 HQ998063 HQ998109 HQ998339 HQ998398
Ploceus bojeri	1	1	HQ998440
Ploceus capensis	1	1	HQ998445
Ploceus castanops	5	4	HQ998046 HQ998170 HQ998181 HQ998312
Ploceus cucullatus	27	14	HQ998034 HQ998035 HQ998044 HQ998045 HQ998085 HQ998086 HQ998096 HQ998097 HQ998250 HQ998260 HQ998301 HQ998358 HQ998393 HQ998437
Ploceus dichrocephalus	2	2	HQ998435 HQ998436
Ploceus dorsomaculatus	2	2	HQ998065 HQ998066

APPENDIX. Continued.

Species	Ns.	Nsq.	Accession numbers
Ploceus flavipes	4	4	HQ998069 HQ998213 HQ998320 HQ998377
Ploceus galbula	1	1	HQ998329
Ploceus grandis	1	1	HQ998360
Ploceus heuglini	3	3	HQ998158 HQ998235 HQ998285
Ploceus insignis	8	7	HQ998014 HQ998061 HQ998062 HQ998211 HQ998308 HQ998348 HQ998396
Ploceus intermedius	4	4	HQ998166 HQ998311 HQ998351 HQ998446
Ploceus jacksoni	4	4	HQ998178 HQ998179 HQ998182 HQ998193
Ploceus katangae	5	5	HQ998037 HQ998038 HQ998154 HQ998155 HQ998187
Ploceus luteola	4	4	HQ998125 HQ998157 HQ998183 HQ998310
Ploceus melanocephalus	10	10	HQ998030 HQ998031 HQ998047 HQ998095 HQ998325 HQ998338 HQ998347 HQ998401 HQ998426 HQ998431
Ploceus melanogaster	5	5	HQ998087 HQ998104 HQ998108 HQ998111 HQ998112
Ploceus nelicourvi	1	1	HQ998039 HQ998040
Ploceus nigerrimus	4	4	HQ998251 HQ998256 HQ998261 HQ998113
Ploceus nigricollis	14	8	HQ998099 HQ998173 HQ998258 HQ998384 HQ998385 HQ998386 HQ998387 HQ998388
Ploceus ocularis	3	3	HQ998098 HQ998342 HQ998395
Ploceus pelzelni	6	5	HQ998028 HQ998059 HQ998340 HQ998364 HQ998430
Ploceus philippinus	1	1	HQ998114
Ploceus preussi	2	2	HQ998064 HQ998429
Ploceus rubiginosus	4	3	HQ998373 HQ998375 HQ998438
Ploceus ruweti	3	2	HQ998144 HQ998447
Ploceus sanctithomae	1	1	HQ998359
Ploceus sp.	4	4	HQ998129 HQ998174 HQ998177 HQ998392
Ploceus spekei	2	2	HQ998349 HQ998434
Ploceus spekeoides	1	1	HQ998379
Ploceus subpersonatus	1	1	HQ998370
Ploceus superciliosus	9	1	HQ998132
Ploceus taeniopterus	6	6	HQ998051 HQ998052 HQ998053 HQ998208 HQ998295 HQ998402
Ploceus tricolor	6	5	HQ998024 HQ998025 HQ998070 HQ998337 HQ998423
Ploceus upembae	3	2	HQ998191 HQ998374
Ploceus velatus	13	12	HQ998032 HQ998043 HQ998149 HQ998171 HQ998172 HQ998188 HQ998207 HQ998249 HQ998343 HQ998442 HQ998443 HQ998444
Ploceus vitellinus	7	7	HQ998041 HQ998042 HQ998078 HQ998079 HQ998080 HQ998169 HQ998195
Ploceus weynsi	3	2	HQ998319 HQ998322
Ploceus xanthops	4	4	HQ998033 HQ998075 HQ998345 HQ998400
Podica senegalensis	1	1	HQ998023
Prionops alberti	1	0	n.a.
Prionops plumata	2	2	HQ998009 HQ998010
Prionops poliocephala	1	1	HQ998011

APPENDIX. Continued.

Species	Ns.	Nsq.	Accession numbers
Prodotiscus insignis	6	5	HQ997956 HQ998119 HQ998217 HQ998291 HQ998314
Prodotiscus regulus	8	7	HQ997957 HQ997958 HQ997959 HQ998131 HQ998147 HQ998231 HQ998264
Prodotiscus zambesiae	4	3	HQ998121 HQ998146 HQ998200
Pseudoalcippe abyssiscicus	3	3	HQ997981 HQ997982 HQ997983
Pseudocalyptomena graueri	2	1	HQ998244
Pseudochelidon eurystomina	3	1	HQ998297
Pteronetta hartlaubii	1	0	n.a.
Pyrenestes ostrinus	3	2	HQ998021 HQ998022
Quelea cardinalis	2	0	n.a.
Quelea erythrops	1	0	n.a.
Quelea quelea	9	6	HQ998019 HQ998020 HQ998068 HQ998102 HQ998352 HQ998354
Quelea sp.	2	2	HQ998084 HQ998094
Riparia congica	2	1	HQ998218
Riparia paludicola	1	0	n.a.
Sagittarius serpentarius	1	0	n.a.
Salpornis spilonotus	2	2	HQ997975 HQ998058
Sarothrura rufa	2	2	HQ997938 HQ997939
Sasia africana	1	1	HQ998100
Schoutedenapus myoptilus	3	0	n.a.
Schoutedenapus schoutedeni	2	0	n.a.
Scopus umbretta	1	0	n.a.
Seicercus laurae	3	3	HQ997989 HQ997990 HQ997991
Smithornis capensis	2	2	HQ997980 HQ998091
Smithornis rufolateralis	1	1	HQ998107
Smithornis sharpei	1	1	HQ998106
Stactolaema anchietae	3	2	HQ997967 HQ997968
Terpsiphone batesi	4	2	HQ998134 HQ998296
Terpsiphone bedfordi	3	3	HQ998229 HQ998265 HQ998270
Terpsiphone mutata	1	0	n.a.
Terpsiphone rufocinerea	2	1	HQ998299
Terpsiphone viridis	3	2	HQ998221 HQ998278
Trochocercus nitens	2	0	n.a.
Turtur brehmeri	1	1	HQ998090
Uraeginthus angolensis	3	2	HQ998015 HQ998017
Uraeginthus bengalus	3	3	HQ998016 HQ998018 HQ998060
Urotriorchis macrourus	1	0	n.a.
Zoothera graueri	1	0	n.a.
Zoothera guttata	1	0	n.a.
Zoothera oberlaenderi	2	1	HQ998165
Zoothera piaggiae	2	0	n.a.
Zosterops maderaspatana	3	0	n.a.